

WestminsterResearch

<http://www.westminster.ac.uk/westminsterresearch>

Comprehensive metabolite profiling of Plantaginis Semen using ultra high performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry coupled with elevated energy technique

Wang, D., Qi, M., Yang, Q., Tong, R, Wang, R, Bligh, S.W.A., Yang, L. and Wang, Z.

This is the peer reviewed version of the following article: Wang, D., Qi, M., Yang, Q., Tong, R, Wang, R, Bligh, S.W.A., Yang, L. and Wang, Z. (2016) Comprehensive metabolite profiling of Plantaginis Semen using ultra high performance liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry coupled with elevated energy technique, *Journal of Separation Science*, 39 (10), pp. 1842-1852, which has been published in final form at

<https://dx.doi.org/10.1002/jssc.201501149>.

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

The WestminsterResearch online digital archive at the University of Westminster aims to make the research output of the University available to a wider audience. Copyright and Moral Rights remain with the authors and/or copyright owners.

Whilst further distribution of specific materials from within this archive is forbidden, you may freely distribute the URL of WestminsterResearch: (<http://westminsterresearch.wmin.ac.uk/>).

In case of abuse or copyright appearing without permission e-mail repository@westminster.ac.uk

**Comprehensive metabolite profiling of Plantaginis Semen using ultra
high performance liquid chromatography with electrospray
ionization quadrupole time-of-flight tandem mass spectrometry
coupled with elevated energy technique**

Dandan Wang¹, Meng Qi¹, Qiming Yang¹, Renchao Tong¹, Rui Wang¹, S.W. Annie
Bligh³, Li Yang^{1, 2*}, Zhengtao Wang¹

Running title: Metabolite profiling of Plantaginis Semen in three species

¹*The Ministry of Education (MOE) Key Laboratory for Standardization of Chinese
Medicines and the STACM Key Laboratory for New Resources and Quality Evaluation
of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of
Traditional Chinese Medicine, shanghai, china*

²*Center for Chinese Medical Therapy and Systems Biology, Shanghai University of
Traditional Chinese Medicine, Shanghai, China.*

³*Department of Life Sciences, Faculty of Science and Technology, University of
Westminster, London W1W 6UW, UK*

Correspondence: Li Yang, Institute of Chinese Materia Medica, Shanghai University
of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201210, China.

E-mail: yangli7951@hotmail.com and y17@shutcm.edu.cn ; Fax: +8651322519; Tel:
+8651322506.

1 Abstract

2 Plantaginis Semen is commonly used in traditional medicine to treat edema,
3 hypertension, and diabetes. The commercially available Plantaginis Semen in China
4 mainly come from three species. In order to clarify the chemical composition and
5 distinct different species of Plantaginis Semen, we established a metabolite profiling
6 method based on ultra high performance liquid chromatography with electrospray
7 ionization quadrupole time-of-flight tandem mass spectrometry coupled with elevated
8 energy technique. A total of 108 compounds, including phenylethanoid glycosides,
9 flavonoids, guanidine derivatives, terpenoids, organic acids, and fatty acids,
10 were identified from *Plantago asiatica* L., *P. depressa* Willd., and *P. major* L.. Results
11 showed significant differences in chemical components among the three species,
12 particularly flavonoids. This study is the first to provide a comprehensive chemical
13 profile of Plantaginis Semen, which could be involved into the quality control,
14 medication guide, and developing new drug of *Plantago* seeds.

15 **Keywords:** mass spectrometry; metabolite profiles; Plantaginis Semen; traditional
16 Chinese medicines; ultra high performance liquid chromatography;.

1 **Abbreviations**

- 2 FAs, fatty acids; MS^E, Elevated Energy mass spectrometry; PAL, *Plantago asiatica* L.;
3 PDW, *Plantago depressa* Willd.; PhG, phenylethanoid glycoside; PML, *Plantago*
4 *major* L.; RDA, retro-Diels–Alder

1 1 Introduction

2 Plantaginis Semen has been traditionally used as medicines and supplements to treat
3 antipyretic, diuretic, and expectorant, etc, in many countries [1]. Previous studies show
4 that Plantaginis Semen contains phenylethanoid glycosides (PhGs), iridoids,
5 flavonoids, and triterpenes [2,3], which account for a variety of pharmaceutical
6 functions such as immunomodulatory, antioxidant, reducing blood lipids and sugar,
7 facilitating defecation, and decreasing blood pressure [4–7]. The sources of
8 commercially available Plantaginis Semen in China mainly consist of *Plantago*
9 *asiatica* L. (PAL), *Plantago depressa* Willd. (PDW), and *Plantago major* L. (PML)
10 [8]. Among them, PAL and PDW are the official sources of Plantaginis Semen in
11 Chinese Pharmacopoeia to treat acute glomerulonephritis, hypertension and liver injury
12 [9]. PML, one of the most abundant and widely distributed medicinal herb in the world,
13 is recorded to exhibit wound healing, anti-inflammatory, antioxidant and antibiotic
14 activities [10, 11]. Identification of Plantaginis Semen from different species is
15 significant for guiding medicine use. However, the authentication is fairly complicated
16 and time-consuming because the seeds are extremely small and have subtle differences
17 on morphological characteristics. As the result, the explanation of chemical
18 constitutions and differences is an effective way to distinguish different species of
19 Plantaginis Semen.

20 Chromatographic fingerprint is a powerful technique and has been widely applied
21 in origin authentication and QC of traditional Chinese medicines. The metabolite
22 profiles reveal chemical compositions of the herbal medicines and contribute to
23 elucidate the pharmacodynamic substance. Various techniques such as
24 photocolormetry, spectrophotometry, HPLC, GC–MS, and LC–MS have been applied
25 to analyze chemical constituents of herbal medicines [12–14]. With recent
26 advancements, UHPLC–ESI–QTOF–MS is considered as a powerful and reliable tool
27 to identify compounds [15–17]. It can provide accurate ion mass values and molecular
28 formulas to elucidate the compound structures [18, 19]. Recently, a novel elevated
29 energy mass spectrometry (MS^E) technique has been utilized to obtain the parent and
30 fragment ion information in a single run by simultaneously acquiring the accurate mass
31 values at high and low collision energies [20, 21]. Combined with MS^E , UHPLC–ESI–
32 QTOF–MS can rapidly provide outstanding chromatographic separation, accurate MS

1 and MS/MS data, and greatly increases the efficiency for compound identification in
2 herbal medicines [22–24].

3 In the present work, a metabolite profiling was established by UHPLC–ESI-
4 QTOF-MS coupled with MS^E technique to clarify the chemical composition
5 Plantaginis Semen from species of PAL, PDW, and PML. Compounds were identified
6 using accurate masses of pseudo-molecular and fragment ions and chromatographic
7 behavior data. Differences of chemical components among the three species were also
8 analyzed, which could be the guidance for medical applications of Plantaginis Semen.

9 **2 Materials and methods**

10 **2.1 Reagents and chemicals**

11 HPLC-grade acetonitrile, methanol, and formic acid were purchased from Fisher
12 Scientific (Santa Clara, USA). Ultrapure water was prepared using a Millipore Alpha-
13 Q water system (Millipore, USA). All other reagents and solvents were of analytical or
14 HPLC grade. Aucubin, geniposidic acid, plantamajoside, rhoifolin, luteolin, and
15 isoquercitrin-7-*O*-gentiobioside were obtained Shanghai R&D Center for
16 Standardization of Chinese Medicines (Shanghai, China). Acteoside, isorhamnetin-3-
17 *O*-glucoside, caffeic acid, eriodictyol, kaempferol, and isorhamnetin were purchased
18 from Meilun Biotech (Dalian, China). Linolenic acid, linolic acid, palmitic acid, and
19 oleic acid were procured from Sigma–Aldrich (St. Louis, USA). Isoacteoside, 2-
20 hydroxyacteoside, plantagoganidinic acid, and plumbagine D were isolated
21 from PAL in our laboratory. The structure was confirmed through MS, ¹³C NMR,
22 and ¹H NMR methods, and purity was over 95% after determined by HPLC–UV.

23 **2.2 Plant materials**

24 A total of 18 batches of Plantaginis Semen were acquired from different provinces in
25 China (Table S1). All of the voucher specimens were authenticated
26 as PAL, PDW, or PML by Professor Li-hong Wu (Shanghai R&D Center for
27 Standardization of Chinese Medicines).

28 **2.3 Sample preparation**

29 All of the samples were pulverized into fine powder. Three hundred mg of powder
30 samples were accurately weighed, dispersed in 20 mL of 60% v/v methanol/water
31 solution, and ultrasonically extracted in a water bath for 30 min at room temperature.

1 The mixtures were then centrifuged at $6000 \times g$ for 10 min and the supernatants were
2 filtered through a $0.22 \mu\text{m}$ filter membrane for analysis.

3 **2.4 Chromatographic conditions**

4 Chromatographic separations were performed on an Acquity UPLC system (Waters,
5 USA) equipped with a binary solvent delivery system and an autosampler. The extracts
6 were separated using an Acquity UPLC BEH C_{18} RP column ($1.7 \mu\text{m}$, $100 \text{ mm} \times 2.1$
7 mm i.d.; Waters, USA) in which the column temperature was maintained at 45°C to
8 avoid excessive column pressure. The mobile phase consisted of 0.1% formic acid in
9 deionized water (mobile phase A) and acetonitrile (mobile phase B). Separation was
10 conducted with the following gradient elution at a flow rate of 0.3 mL/min : 0–1 min,
11 5% B; 1–4 min, 5–15% B; 4–5 min, 15–17% B; 5–7 min, 17% B; 7–9 min, 17–23%
12 B; 9–14 min, 23–50% B; 14–23 min, 50–65% B; 23–28 min, 65–95% B, and 28–30
13 min, 5% B for equilibration of the column. The injection procedure was carried out for
14 1 min. An aliquot of $5 \mu\text{L}$ of sample solution was injected for analysis.

15 **2.5 Mass spectrometric conditions**

16 MS detection was performed using Acquity Synapt G2 QTOF tandem mass
17 spectrometer (Waters, UK) connected to the UHPLC system by an ESI interface and
18 controlled by MassLynx version 4.1 (Waters, UK). The ESI source was operated in
19 both positive (ESI+) and negative (ESI-) ionization modes. The optimized conditions
20 to trigger maximum response of metabolites were listed as follows: capillary voltage,
21 -2.5 kV (ESI-) or $+3 \text{ kV}$ (ESI+); sample cone, -25 V (ESI-) or $+30 \text{ V}$ (ESI+);
22 extraction cone, -4.0 V (ESI-) or $+4.0 \text{ V}$ (ESI+); source temperature, 120°C ;
23 desolvation temperature, 350°C ; cone gas (nitrogen) flow, 50 L/h ; and desolvation gas
24 (nitrogen) flow, 600 L/h . Argon was used as collision gas. Leucine-enkephalin (2
25 ng/mL) was used as the lock mass generating a reference ion at m/z of 554.2615 (ESI-)
26 or 556.2771 (ESI+) by a lockspray at $5 \mu\text{L/min}$ to acquire accurate mass during
27 analysis.

28 Data were collected in a centroid mode. MS^E approach was conducted with two
29 scan functions. In function 1, the following parameters were set: m/z $50\text{--}1500$; scan
30 duration, 0.3 s ; interscan delay, 0.024 s ; and collision energy ramp, 4 V . In function 2,
31 the following parameters were set: m/z $50\text{--}1500$; scan duration, 0.3 s ; interscan delay,
32 0.024 s ; and collision energy ramp, $10\text{--}30 \text{ V}$. In MS^E , MS and MS/MS data can be

1 acquired almost simultaneously in a single analytical run. Data acquisition and
2 processing were conducted using Waters MassLynx version 4.1.

3 **3 Results and Discussion**

4 **3.1 Sample preparation and metabolite profiling**

5 Ultrasonication was selected for the extraction for its time-saving, convenient, and
6 reproducible. Methanol/water ratio was optimized to 60% v/v to achieve the maximum
7 extraction efficiency of compounds with different polarities. In UHPLC–ESI-QTOF-
8 MS analysis, gradient elution, flow rate, and column temperature were evaluated for an
9 optimized chromatographic condition. For a comprehensive analyses of the metabolite
10 compositions of the extracts, positive and negative ionization ESI modes were used to
11 verify the molecular formula of online MW assignments of the unknown substances.

12 **3.2 Identification strategy**

13 A total of 108 compounds belonging to phenylethanoid glycosides (PhGs), guanidine
14 derivatives, flavonoids, terpenoids, organic acids, fatty acids (FAs) were identified
15 (Table S2). Chromatograms of three different species of *Plantaginis Semen* in negative
16 ion mode are shown in Fig. 1. The UHPLC–ESI-QTOF-MS coupled with
17 MS^E technique could simultaneously afford accurate mass values of precursor and
18 fragment ions in a single injection. The detected peaks were then identified by their
19 elemental compositions or comparing MS^E data to the literatures or public online
20 databases, including PubChem (<http://pubchem.ncbi.nlm.nih.gov>), ChemSpider
21 (<http://www.chemspider.com>), Kegg Ligand Database
22 (<http://www.genome.jp/kegg/ligand.html>), SciFinder Scholar
23 (<https://scifinder.cas.org>), Metlin (<http://metlin.scripps.edu>), and Riken
24 (<http://spectra.psc.riken.jp/menta.cgi/index>). Mass errors between measured and
25 calculated values were <1.2 mDa or 5 ppm to guarantee high resolution and good
26 accuracy. We also use 20 standard compounds, including one organic acid, two iridoids,
27 two guanidine derivatives, four fatty acids, seven flavonoids, and four phenylethanoid
28 glycosides to confirm the identification results. Furthermore, these standards were
29 applied to evaluate the proposed method which was proved to be stable and reliable.

30 **3.3 Chemical constituents in *Plantaginis Semen***

31 *Phenylethanoid glycosides*

PhGs are natural products widely distributed in the plant kingdom and isolated from many medicinal plants [25]. PhGs exhibit verifiable therapeutic effects, including neuroprotection, antioxidation, anti-metastasis and cytotoxicity [26–28]. In this study, 11 PhGs were characterized in *Plantaginis Semen*, shown in Table 1. Among them, 2-hydroxyacteoside, plantamajoside, acteoside, and isoacteoside were identified and confirmed by comparing with standards. According to the PhGs determined in this experiment, the central glucose always connects with rhamnose by Rha (1→3) Glu linkage. Glucose is also directly attached to aglycone, and caffeoyl is usually located at C₄ or C₆ position of glucose. The detected PhGs produced similar fragmentation patterns in the MS^E spectra. The neutral losses of 162, 152, or 146 Da were related to caffeic acid, glucose, phenethanol aglycone, and rhamnose. H₂O or CO₂ is frequently eliminated in the fragmentations. Identical product ions at *m/z* 179.0369, 161.0235, and 135.0457 were observed, which respectively indicated the presence of caffeoyl, anhydroglucose, and anhydrophenylethanol. We take plantamajoside to illustrate the fragmentation pathway of PhGs (Fig. 2). The molecular formula of plantamajoside was C₂₉H₃₆O₁₆ with *m/z* 639.1944 [M–H][–]. In the MS^E spectrum, the fragment ion at *m/z* 477.1632 was formed by eliminating caffeoyl residue from the precursor ion. The fragment ion at *m/z* 315.1056 was from the group of central glucose with phenethanol aglycon. Furthermore, two fragment ions at *m/z* 179.0369 and 161.0235 could be served as characteristic ions to identify PhGs, which are respectively produced from the dehydrogenation and the dehydration of caffeic acid.

Flavonoids

Flavonoids are a chemically and structurally diverse group of polyphenolic compounds widely distributing in all parts of the plant [29]. Flavonoids are recognized as pigments responsible for leaf colors and are mainly involved in biological processes, such as the protection of plant tissues against UV radiation [30, 31].

A total of 23 flavonoids, including ten flavonols, four flavones, two flavanonols, six flavanones, and one aurone, were identified in this study (Table 2); most of these compounds are present in the form of glycosides with sugars attached to flavonoid aglycone by C–O bonds. Among them, isoquercitrin-7-*O*-gentiobioside, isorhamnetin-3-*O*-glucoside, rhoifolin, eriodictyol, luteolin, kaempferol, and isorhamnetin were verified using reference standards. Flavonoids mainly consist of two aromatic

1 benzene rings separated by an oxygenated heterocyclic ring. The characteristic
2 fragmentation of flavonoid aglycones involves RDA cleavage of ring C and multiple
3 neutral loss pathways, such as sequential elimination of sugar residues [32].
4 Compound **37** with quasi-molecular ion at m/z 465.1040 $[M-H]^-$ indicated the
5 molecular formula of $C_{21}H_{22}O_{12}$. In the MS^E spectrum, compound **37** displayed
6 fragment ions at m/z 313.0919, 303.0500 and 151.0021, suggesting the loss of glucose
7 and the RDA cleavage of ring C. Compound **37** was finally identified as plantagoside.
8 Compound **47** was identified as pentahydroxyflavanone. The $[M-H]^-$ ion
9 of compound **47** was m/z 303.0500 with the elemental composition of $C_{15}H_{11}O_7$. The
10 MS^E result showed that the product ions at m/z 151.0023 and 107.0120 respectively
11 corresponded to the RDA cleavage of ring C and the loss of CO_2 , indicating that
12 compound **47** was the aglycone of plantagoside (Fig. 3). Plantagoside and
13 pentahydroxyflavanone were first isolated from *P.*
14 *asiatica* var. *japonica* [33] and *Helichrysum bracteatum* [34]. These compounds
15 inhibit the formation of advanced glycation end products of proteins under
16 physiological conditions and impede protein cross-linking glycation [35].

17 Compounds **19** and **27** (ampelopsin glucoside) are the characteristic components
18 of PDW. Ampelopsin is one of the most common flavonoids involved in multiple
19 biological activities, such as antimicrobial and antioxidant effects, as well as
20 antihypertension and hepatoprotection [36, 37]. Ampelopsin glucosides exhibit more
21 efficient antioxidant properties than ampelopsin alone [38].
22 Compounds **19** and **27** were tentatively identified as ampelopsin-4'-glucoside and
23 ampelopsin-3'-glucoside, respectively, based on the MS^E fragmentation pattern.

24 ***Guanidine derivatives***

25 Guanidine derivatives are a group of infrequent alkaloids which are mostly reported in
26 marine organisms [39]. This group of compounds is widely applied in medicine,
27 chemistry, and other industries because of their strong alkalinity, high stability, and
28 good biological activities [40, 41]. Guanidine derivatives ionize more efficiently in the
29 positive ion mode. In this study, we characterized 18 guanidine derivatives which
30 contained similar imidazoline skeletons (Table 3). The structures of plantagoguanidinic
31 acid and plumbagine D were confirmed by standard compounds. Plantagoguanidinic
32 acid was first isolated from PAL seeds [42]. The fragment ion of plantagoguanidinic

1 acid at m/z 208.1445 was generated by the elimination of H_2O from the carboxyl (Fig.
2 4). The product ion at m/z 84.0562 was from the cleavage between the imidazoline ring
3 and the side chain. Plumbagine D, first discovered from *Plumbago zeylanica* [43], has
4 one more butanediol group replaced on the imidazoline ring than plantagoganidinic
5 acid. Plumbagine D then yielded fragment ions at m/z 226.1567 and 172.1093 from the
6 side-chain cleavage and the specific product ion at m/z 84.0567 from imidazoline ring
7 (Fig. 4). These fragmentation pattern can be applied to characterize the remaining
8 unknown guanidine derivatives. For example, the $[M+H]^+$ ion of compound **11** with the
9 molecular formula of $C_{15}H_{28}N_3O_5$ was detected at m/z 330.2028. The MS^E spectrum
10 showed that a H_2O loss was generated at m/z 312.1938. Compound **11** also displayed
11 the same fragment ions at m/z 172.1082 and 84.0576 as plumbagine D. The results
12 demonstrated that compound **11** was hydroxy-substituted plumbagine D, and was
13 tentatively recognized as plumbagine E [43].

14 **Terpenoids**

15 Five iridoids and four triterpenoids were characterized from Plantaginis Semen (Table
16 4). Iridoids are a group of terpene-derived compounds which have structural similarity
17 and biosynthetic relationship to iridodial and iridomyrmecin [44]. Plenty of iridoids
18 have been isolated from the genus *Plantago*. Iridoids in this experiment displayed
19 strong MS response in negative ion mode. In the spectrum of geniposidic acid, fragment
20 ion at m/z 211.0607 was obtained after the elimination of glucose residue. Further
21 fragment ions at m/z 123.0443 and 89.0236 were also exhibited because of $^{1,4}F$
22 cleavage. Furthermore, the neutral loss of CO_2 occurred in this compound to indicate
23 the presence of a carboxyl functionality. Aucubin generated specific solvent adducts
24 ion m/z 391.1245 $[M+HCOO]^-$ with high intensity and underwent similar cleavage
25 pathways as geniposidic acid. The other three iridoids (8-epiloganic acid,
26 gentiopicroside, and catalposide) were also identified by similar cleavage pathways.

27 Triterpenoids have been recognized to have hepatoprotective, antihyperlipidemic,
28 anticancer, and anti-inflammatory effects. These compounds are synthesized from
29 isopentenyl pyrophosphate by the 30-carbon intermediate squalene. In this study, four
30 triterpenoids (sumaresinol, oleanolic acid, ursonic acid, and oleanolic acid acetate)
31 were identified by comparing with those described in previous studies [45, 46].

32 **Organic acids and amino acids**

Four organic acids (gluconic acid, citric acid, ferulic acid, and caffeic acid) and one amino acid (tryptophan) were identified in *Plantaginis Semen* (Table S2). Organic acids and amino acids are a widespread primary metabolites that play essential roles in plant growth processes, including respiration, photosynthesis, and hormone and protein syntheses [47]. In the MS/MS fragmentation, organic acids generate the neutral losses of CO₂ from the carboxylic group or H₂O, and amino acids generate neutral losses of CO₂ and the amino group NH₃.

Fatty acids

FAs are essential macromolecules present in all living organisms. FAs consist of long hydrophobic, often unbranched chains of hydrocarbons, with hydrophilic carboxylic acid groups at one end [48]. FAs and their derivatives also function as signaling molecules that modulate normal and disease-related physiological characteristics in microbes, insects and other animals, and plants [49].

We identified 28 FAs in the extracts of *Plantaginis Semen* (Table S2). Among them, linolenic acid, linolic acid, palmitic acid, and oleic acid were previously reported in PAL and then confirmed by comparing with authentic standards. The other detected FAs were C16 and C18 hydroxy FAs, which are presumably produced by oxidative metabolism of polyunsaturated FAs. However, the exact structures of four trihydroxyoctadecadienoic acids and three hydroxyoctadecatrienoic acids could not be defined for the uncertain locations of hydroxy groups and double bonds.

3.4 Comparison of the metabolite profiles of three *Plantago* species

We had collected 18 batches of *Plantaginis Semen* in species of PAL, PDW and PML from different places in China, in which PAL and PDW are the official sources recorded in Chinese Pharmacopoeia. The UHPLC–ESI-QTOF-MS Chromatograms revealed obvious diversities in chemical composition of three *Plantago* species (Fig. S1). PhGs and iridiods are primary bioactive components of *Plantago* species and acteoside and geniposidic acid are recognized as the QC markers of *Plantaginis Semen* in Chinese Pharmacopoeia [6, 9]. In our study, acteoside displayed similar levels in PAL and PDW (Table 1), while the amount of geniposidic acid in PAL is higher than in PDW (Table 4). Moreover, PML displayed low levels of PhGs and terpenoids. The contents of acteoside and geniposidic acid in PML were significantly lower than PAL and PDW and did not meet the quality standards for *Plantaginis Semen*. The results indicated that

1 according to the test standards for Plantaginis Semen in Chinese Pharmacopoeia, the
2 quality of the seeds from PAL was superior to those from PDW and PML was
3 unqualified to serve as the source of Plantaginis Semen.

4 Different flavonoid levels were also found in PAL, PDW and PML (Table 2). In
5 General, PML contains the greatest amount of flavonoids followed by PDW and PAL.
6 Meanwhile, PML had more widely spread of flavonoids. Therefore the seeds of PML
7 probably have advantages in anti-oxidative, anti-inflammatory and anti-bacterial
8 effects [35–38]. In addition, the three species of Plantaginis Semen could be
9 differentiated by several flavonoids. Plantagoside, isoquercitrin, and
10 pentahydroxyflavanoneare were much higher in PML than in PAL and PDW.
11 Ampelopsin glucoside and its isomer showed high concentrations in PDW while could
12 not be determined in PML.

13 Guanidine derivatives are a group of novel alkaloids which have potential
14 hypoglycemic effect [42]. The three species of Plantaginis Semen showed similar
15 contents of Plantagoguanidinic acid and Plumbagine D, while PAL and PML displayed
16 higher amounts of total guanidine derivatives (Table 3). In that case, PAL and PML
17 could be used as the resources for guanidine separation, which is greatly helpful for the
18 development of new glucose-lowering drugs.

19 Furthermore, PAL contained higher amounts of FAs than PDW and PML. Previous
20 reports showedthat FAs display antioxidation, immunoregulation, vascular-protection,
21 and cholesterol-reducing effects [50]. For that reason, the seeds of PAL might be the
22 first choice to treat metabolic diseases.

23 **4 Conclusions**

24 In the present study, the metabolite profiling based on UHPLC–ESI-QTOF-MS
25 combined with MS^E technique was applied to evaluate the chemical variation of three
26 species of Plantaginis Semen, PAL, PDW, and PML. In this experiment, MS^E technique
27 provided accurate mass values of precursor and fragment ions in a single injection.
28 Positive and negative ionization modes were applied, and the chemical formula was
29 obtained based on the errors <1.2 mDa or 5 ppm between measured and calculated mass
30 values. Twentystandard compounds were collected to validate the reliability of the
31 proposed method. Using this approach, we identified 108 compounds and most of them
32 were first detected in Plantaginis Semen. Our results indicated significant differences

1 in chemical compositions among the three species. PAL showed higher levels of
2 PhGs, terpenoids, guanidine derivatives and FAs, while PML contained the greatest
3 amount of flavonoids. This study provides a comprehensive chemical profile of
4 Plantaginis Semen, which could be involved into the QC, medication guide, and
5 developing new drug of Plantago seeds.

6 **Acknowledgements**

7 The authors gratefully acknowledge the financial support from the National S&T
8 Major Special Projects (2012ZX09103201-045), the National Natural Science
9 Foundation of China (81222053 and 81403070), the Program for New Century
10 Excellent Talents in University (NCET-12-1056), China Postdoctoral Science
11 Foundation (2014M551438), the “Shu-Guang Scholar” Project (11SG41) and the
12 Budget Project (2013JW21) of Shanghai Municipal Education Commission.

1 References

- 2 [1] Doan D. D., Nguyen N. H., Doan H. K., Nguyen T. L., Phan T. S., Van Dau N.,
3 Grabe M., Johansson R., Lindgren G., Stjernström N. E., Studies on the individual and
4 combined diuretic effects of four Vietnamese traditional herbal remedies (*Zea*
5 *mays*, *Imperata cylindrica*, *Plantago major* and *Orthosiphon stamineus*).
6 J. Ethnopharmacol. 1992, 36, 225-231.
- 7 [2] Qi M., Xiong A. Z., Geng F., Yang L., Wang Z. T., A novel strategy for target
8 profiling analysis of bioactive phenylethanoid glycosides in *Plantago* medicinal plants
9 using ultra-performance liquid chromatography coupled with tandem quadrupole mass
10 spectrometry. J. Sep. Sci. 2012, 35, 1470-1478 [PubMed](#) .
- 11 [3] Nhiem N. X., Tai B. H., Van Kiem P., Van Minh C., Cuong N. X., Tung N. H.,
12 Thu V. K., Trung T. N., Anh Hle T., Jo S. H., Jang H. D., Kwon Y. I., Kim Y. H.,
13 Inhibitory activity of *Plantago major* L. on angiotensin I-converting enzyme. Arch.
14 Pharm. Res. 2011, 34, 419-423.
- 15 [4] Huang D. F., Xie M. Y., Yin J. Y., Nie S. P., Tang Y. F., Xie X. M., Zhou C.,
16 Immunomodulatory activity of the seeds of *Plantago*
17 *asiatica* L.. J. Ethnopharmacol. 2009, 124, 493-498.
- 18 [5] Harput U. S., Genc Y., Saracoglu I., Cytotoxic and antioxidative activities
19 of *Plantago lagopus* L. and characterization of its bioactive compounds. Food
20 Chem. Toxicol. 2012, 50, 1554-1559 [PubMed](#) .
- 21 [6] Zhou Q., Lu W., Niu Y., Liu J., Zhang X., Gao B., Akoh C. C., Shi H., Yu L. L.,
22 Identification and quantification of phytochemical composition and anti-inflammatory,
23 cellular antioxidant, and radical scavenging activities of 12 *Plantago* species. J. Agric.
24 Food Chem. 2013, 61, 6693-6702.
- 25 [7] Choi S. Y., Jung S. H., Lee H. S., Park K. W., Yun B. S., Lee K. W., Glycation
26 inhibitory activity and the identification of an active compound in *Plantago*
27 *asiatica* extract. Phytother. Res. 2008, 22, 323-329 [PubMed](#) .
- 28 [8] Liu X., Wu X., Huang H., Zhong S., Lai X., Cao L., Herbalogical study on *Plantago*
29 *asiatica* L.. J. Chinese Med. Mater. 2002, 25, 46-48.

- 1 [9] China Pharmacopoeia Committee., China Pharmacopoeia, vol. 1. Chemical Industry
2 Press, Beijing 2010, pp 337-338.
- 3 [10] Zubair M., Nybom H., Lindholm C., Brandner J. M., Rumpunen K., Promotion of
4 wound healing by *Plantago major* L. leaf extracts - ex-vivo experiments confirm
5 experiences from traditional medicine. Nat. Prod. Res. 2016, 30, 622-624 [PubMed](#) .
- 6 [11] Hussan F., Mansor A. S., Hassan S. N., Tengku Nor Effendy Kamaruddin T. N.,
7 Budin S. B., Othman F., Anti-inflammatory property of *Plantago major* leaf extract
8 reduces the inflammatory reaction in experimental acetaminophen-induced liver injury.
9 Evid. Based Complement Alternat. Med. 2015, 2015, 347861.
- 10 [12] Li B. Q., Chen J., Li J. J., Wang X., Zhai H. L., Zhang X. Y., High-performance
11 liquid chromatography with photodiode array detection and chemometrics method for
12 the analysis of multiple components in the traditional Chinese medicine
13 Shuanghuanglian oral liquid. J. Sep. Sci. 2016, 38, 4187– [PubMed](#) ;4195.
- 14 [13] Samuelsen A. B., Cohen E. H., Paulsen B. S., Brüll L. P., Thomas-Oates J.
15 E., Structural studies of a heteroxylan from *Plantago major* L. seeds by partial
16 hydrolysis, HPAEC-PAD, methylation and GC-MS, ESMS and ESMS/MS. Carbohydr.
17 Res. 1999, 315, 312-318.
- 18 [14] Chen L., Jian Y., Wei N., Yuan M., Zhuang X. M., Li H., Separation and
19 simultaneous quantification of nine furanocoumarins from Radix Angelicae dahuricae
20 using liquid chromatography with tandem mass spectrometry for bioavailability
21 determination in rats. J. Sep. Sci. 2016, 38, 4216– [PubMed](#) ;4224.
- 22 [15] Sugimoto T., Bamba T., Izumi Y., Nomura H., Shiina T., Fukusaki E., Use of ultra-
23 performance liquid chromatography/time-of-flight mass spectrometry with nozzle-
24 skimmer fragmentation for comprehensive quantitative analysis of secondary
25 metabolites in *Arabidopsis thaliana*. J. Sep. Sci. 2011, 34, 3587-3596 [PubMed](#) .
- 26 [16] Kim J. Y., Park J. Y., Kim O. Y., Ham B. M., Kim H. J., Kwon D. Y., Jang Y.,
27 Lee J. H., Metabolic profiling of plasma in overweight/obese and lean men using ultra
28 performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF
29 MS). J. Proteome Res. 2010, 9, 4368-4375.
- 30 [17] Xie G. X., Ni Y., Su M. M., Zhang Y. Y., Zhao A. H., Gao X. F., Liu Z., Xiao P.
31 G., Jia W., Application of ultra-performance LC-TOF MS metabolite profiling

- 1 techniques to the analysis of medicinal Panax herbs. *Metabolomics* 2008, 4, 248-
- 2 260 [PubMed](#) .
- 3 [18] Zhang Y., Li F., Huang F., Xie G., Wei R., Chen T., Liu J., Zhao A., Jia W.,
- 4 *Metabolomics analysis reveals variation in Schisandra chinensis* cetabolites from
- 5 different origins. *J. Sep. Sci.* 2014, 37, 731-737 [PubMed](#) .
- 6 [19] Wang F., Ai Y., Wu Y., Ma W., Bian Q., Lee D. Y., Dai R., Systematic chemical
- 7 profiling of a multicomponent Chinese herbal formula Huo Luo Xiao Ling Dan by ultra
- 8 high performance liquid chromatography coupled with electrospray ionization
- 9 quadrupole time-of-flight mass spectrometry. *J. Sep. Sci.* 2015, 38, 917-924 [PubMed](#) .
- 10 [20] Plumb R. S., Jones M. D., Rainville P., Castro-Perez J. M., The rapid detection
- 11 and identification of the impurities of simvastatin using high resolution sub 2 microm
- 12 particle LC coupled to hybrid quadrupole time of flight MS operating with alternating
- 13 high-low collision energy. *J. Sep. Sci.*, 2007, 30, 2666-2675.
- 14 [21] Han H., Xiong A. Z., He C. Y., Liu Q., Yang L., Wang Z. T., Combination of
- 15 UHPLC-Q-TOF-MS, NMR spectroscopy, and ECD calculation for screening and
- 16 identification of reactive metabolites of gentiopicroside in humans. *Anal. Bioanal*
- 17 *Chem.* 2014, 406, 1781-1793 [PubMed](#) .
- 18 [22] Zhao Y. Y., Cheng X. L., Wei F., Bai X., Tan X. J., Lin R. C., Mei Q., Intrarenal
- 19 metabolomic investigation of chronic kidney disease and its TGF- β 1 mechanism in
- 20 induced-adenine rats using UPLC Q-TOF/HSMS/MS(E). *J. Proteome Res.* 2013, 12,
- 21 692-703 [PubMed](#) .
- 22 [23] Qi M., Xiong A., Li P., Yang Q., Yang L., Wang Z., Identification of acteoside
- 23 and its major metabolites in rat urine by ultra-performance liquid chromatography
- 24 combined with electrospray ionization quadrupole time-of-flight tandem mass
- 25 spectrometry. *J. Chromatogr. B* 2013, 940, 77-85 [PubMed](#) .
- 26 [24] Bateman K. P., Castro-Perez J., Wrona M., Shockcor J. P., Yu K., Oballa R.,
- 27 Nicoll-Griffith D. A., MSE with mass defect filtering for *in vitro* and *in vivo* metabolite
- 28 identification. *Rapid Commun. Mass Spectrom* 2007, 21, 1485-1496.
- 29 [25] Jiménez C., Rigüera R., Phenylethanoid glycosides in plants: structure and
- 30 biological activity. *Nat. Prod. Rep.* 1994, 11, 591-606.

- 1 [26] Koo K. A., Sung S. H., Park J. H., Kim S. H., Lee K. Y., Kim Y. C., *In*
2 *vitro* neuroprotective activities of phenylethanoid glycosides from *Callicarpa*
3 *dichotoma*. *Planta Med.* 2005, 71, 788-780.
- 4 [27] Wong I. Y., He Z. D., Huang Y., Chen Z. Y., Antioxidative activities of
5 phenylethanoid glycosides from *Ligustrum purpurascens*. *J. Agr. Food Chem.* 2001,
6 49, 3113-3119.
- 7 [28] Rao Y. K., Lien H., Lin Y., Hsu Y., Yeh C., Chen C., Lai C., Tzeng Y.,
8 Antibacterial activities of *Anisomeles indica* constituents and their inhibition effect
9 on *Helicobacter pylori*-induced inflammation in human gastric epithelial cells. *Food*
10 *Chem.* 2012, 132, 780-787 [PubMed](#) .
- 11 [29] Cook N. C., Samman S., Flavonoids-chemistry, metabolism, cardioprotective
12 effects, and dietary sources. *J. Nutr. Biochem.* 1996, 7, 66-76 [PubMed](#) .
- 13 [30] Middleton E. Jr., Kandaswami C., Theoharides T. C., The effects of plant
14 flavonoids on mammalian cells: implications for inflammation, heart disease, and
15 cancer. *Pharmacol. Rev.* 2000, 52, 673-751 [PubMed](#) .
- 16 [31] Andersen O. M., Markham K. R., Flavonoids: chemistry, biochemistry and
17 applications. CRC Press, Boca Raton 2005.
- 18 [32] Jin X. F., Lu Y. H., Wei D. Z., Wang Z. T., Chemical fingerprint and quantitative
19 analysis of *Salvia plebeia* R.Br. by high-performance liquid
20 chromatography. *J. Pharm. Biomed. Anal.* 2008, 48, 100-104.
- 21 [33] Tohru E., Heihachiro T., Itiro Y., The glycosides of *Plantago*
22 *major* var. *japonica* NAKAI. A new flavanone glycoside, plantagoside. *Chem. Pharm.*
23 *Bull.* 1981, 29, 1000-1004 [PubMed](#) .
- 24 [34] Forkmann G., 5, 7, 3',4', 5'-pentahydroxyflavanone in the bracts of *Helichrysum*
25 *bracteatum*. *Z. Naturforsch. C* 1983, 38, 891-893 [PubMed](#) .
- 26 [35] Matsuura N., Aradate T., Kurosaka C., Ubukata M., Kittaka S., Nakaminami Y.,
27 Gamo K., Kojima H., Ohara M., Potent protein glycation inhibition of plantagoside
28 in *Plantago major* seeds. *Biomed. Res. Int.* 2014, 2014, 208539.

- 1 [36] Zhang Y. S., Ning Z. X., Yang S. Z., Wu H., Antioxidation properties and
2 mechanism of action of dihydromyricetin from *Ampelopsis grossedentata*. Acta Pharm.
3 Sinica 2003, 38, 241-244.
- 4 [37] Zheng H. Q., Liu D. Y., Anti-invasive and anti-metastatic effect of ampelopsin on
5 melanoma. Chinese J. Cancer 2003, 22, 363-367 [PubMed](#) .
- 6 [38] Woo H. J., Kang H. K., Nguyen T. T., Kim G. E., Kim Y. M., Park J. S., Kim
7 D., Cha J., Moon Y. H., Nam S. H., Xia Y. M., Kimura A., Kim D., Synthesis and
8 characterization of ampelopsin glucosides using dextransucrase from *Leuconostoc*
9 *mesenteroides*B-1299CB4: glucosylation enhancing physicochemical
10 properties. Enzyme Microb. Technol. 2012, 51, 311-318.
- 11 [39] Berlinck R. G. S., Braekman J. C., Daloze D., Hallenga K., Ottinger R., Bruno I.,
12 Riccio R., Two new guanidine alkaloids from the mediterranean sponge crambe
13 crambe. Tetrahedron Lett. 1990, 31, 6531-6534.
- 14 [40] Pottabathula S., Royo B., First iron-catalyzed guanylation of amines: a simple and
15 highly efficient protocol to guanidines. Tetrahedron Lett. 2012, 53, 5156-5158.
- 16 [41] Sugimoto H., Iimura Y., Yamanishi Y., Yamatsu K., Synthesis and structure-
17 activity relationships of acetylcholinesterase inhibitors: 1-benzyl-4-[(5,6-dimethoxy-1-
18 oxoindan-2-yl)methyl]piperidine hydrochloride and related
19 compounds. J. Med. Chem. 1995, 38, 4821-4829.
- 20 [42] Goda Y., Kawahara N., Kiuchi F., Hirakura K., Kikuchi Y., Nishimura H., Takao
21 M., Marumoto M., Kitazaki H., A guanidine derivative from seeds of *Plantago*
22 *asiatica*. J. Nat. Med. 2009, 63, 58-60 [PubMed](#) .
- 23 [43] Cong H. J., Zhang S. W., Shen Y., Zheng Y., Huang Y. J., Wang W. Q., Leng Y.,
24 Xuan L. J., Guanidine alkaloids from *Plumbago zeylanica*. J. Nat. Prod. 2013, 76, 1351-
25 1357 [PubMed](#) .
- 26 [44] Bowers M. D., Herbivores: their interactions with secondary plant metabolites.
27 Iridoid glycosides. Academic Press, San Diego 1991, pp 297-325.
- 28 [45] Chen Q., Zhang Y., Zhang W., Chen Z., Identification and quantification of
29 oleanolic acid and ursolic acid in Chinese herbs by liquid chromatography-ion trap
30 mass spectrometry. Biomed. Chromatogr. 2011, 25, 1381-1388.

- 1 [46] Phillips D. R., Rasbery J. M., Bartel B., Matsuda S. P., Biosynthetic diversity in
2 plant triterpene cyclization. *Curr. Opin. Plant Biol.* 2006, 9, 305-314.
- 3 [47] Zelitch I., Organic acids and respiration in photosynthetic tissues. *Annu. Rev. Plant*
4 *Physiol.* 1964, 15, 121-142 [PubMed](#) .
- 5 [48] Kachroo A., Kachroo P., Fatty acid-derived signals in plant defense. *Annu. Review*
6 *Phytopathol.* 2009, 47, 153-176 [PubMed](#) .
- 7 [49] Thelen J. J., Ohlrogge J. B., Metabolic engineering of fatty acid biosynthesis in
8 plants. *Metab. Eng.* 2002, 4, 12-21 [PubMed](#) .
- 9 [50] Ide T., Effect of dietary α -lipoic acid on the mRNA expression of genes involved
10 in drug metabolism and antioxidation system in rat liver. *Br. J. Nutr.* 2014, 112, 295-
11 308 [PubMed](#) .

12

1 **Figure captions**

2 **Fig. 1** Base peak ion chromatograms of three species of Plantaginis Semen obtained
3 by UHPLC-ESI-QTOF-MS in the negative ion mode. (a) PAL, (b) PDW and (c) PML.

4 **Fig. 2** Proposed fragmentation pathway of plantamajoside.

5 **Fig. 3** Proposed fragmentation pathways of plantagoside (a) and
6 pentahydroxyflavanone (b).

7 **Fig. 4** Proposed fragmentation patterns of plantagoguanidinic acid (a) and
8 plumbagine D (b).

Table 1 Contents of PhGs in three species of Plantaginis Semen (mg/g crude drug, mean \pm SE)

No.	Compound	R ₁	R ₂	R ₃	R ₄	R ₅	PAL	PDW	PML
12	Decaffeoyllacteoside	OH	H	0.037 \pm 0.004	—*	-			
34	2-Hydroxyacteoside ^a	Rha	Caffeoyl	H	OH	H	0.056 \pm 0.006	-	-
38	Plantamajoside ^a	Glu	Caffeoyl	H	H	H	0.491 \pm 0.079	-	0.193 \pm 0.074
44	Acteoside ^a	Rha	Caffeoyl	H	H	H	6.334 \pm 0.827	6.423 \pm 1.043	3.606 \pm 0.776
45	Isoplantamajoside	Glu	H	Caffeoyl	H	H	0.183 \pm 0.034	-	-
48	Isoacteoside ^a	Rha	H	Caffeoyl	H	H	1.433 \pm 0.243	0.67 \pm 0.181	0.11 \pm 0.081
Total content							8.924 \pm 1.198	8.114 \pm 1.037	4.361 \pm 1.087

³ ^a This compound is confirmed by standards.

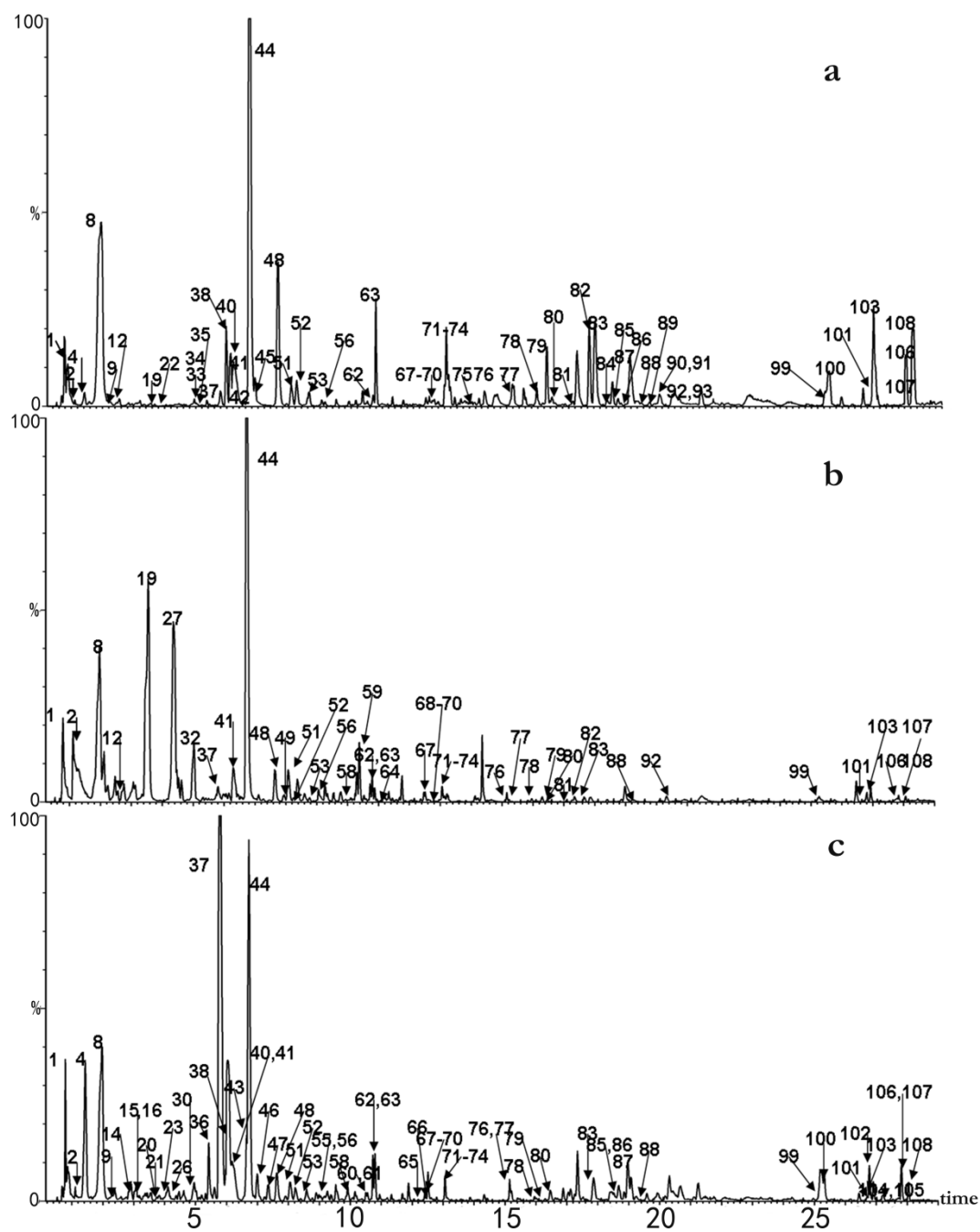
⁴ * (-) indicates absence of the compound.

Table 2 Contents of Flavonoids in three species of Plantaginis Semen (mg/g crude drug, mean \pm SE)

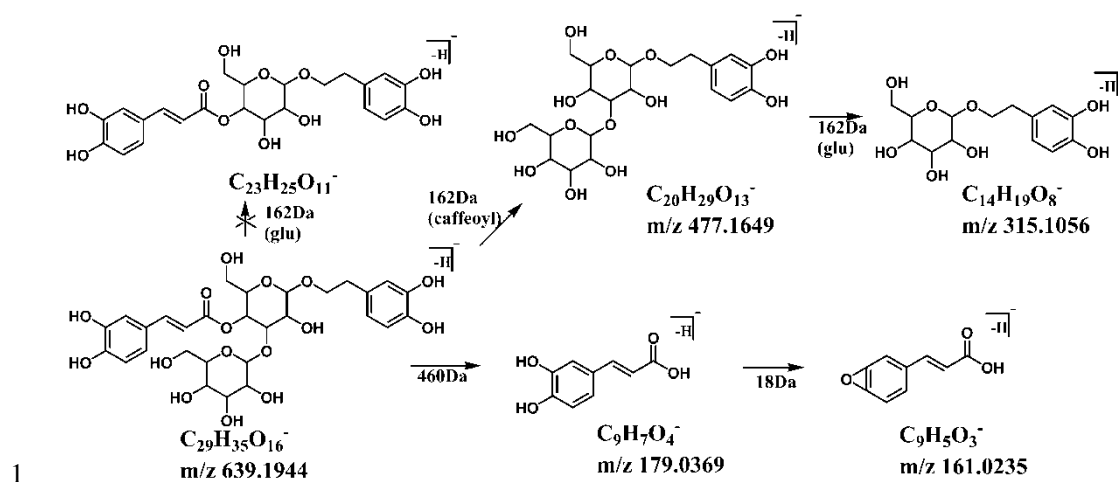
No.	Compound	PAL	PDW	PML
15	Isoquercitrin-7-O-gentiobioside ^a	—*	-	0.459 \pm 0.021
16	Luteoloside dihexose	-	-	0.206 \pm 0.04
19	Ampelopsin glucoside	0.121 \pm 0.022	4.121 \pm 0.450	-
20	Isorhamnetin triglucoside	-	-	0.364 \pm 0.035
21	Isorhamnetin triglucoside isomer	-	-	0.124 \pm 0.028
26	Plantagoside-hexoside I	-	-	0.432 \pm 0.114
27	Ampelopsin glucoside isomer	0.089 \pm 0.017	3.603 \pm 0.259	-
30	Plantagoside-hexoside II	-	-	0.269 \pm 0.057
35	Quercetin 3,7-dihexoside/ Quercetin 3-sophoroside	0.059 \pm 0.008	-	-
37	Plantagoside	0.139 \pm 0.013	0.130 \pm 0.038	9.520 \pm 0.948
39	Hyperoside	-	-	0.063 \pm 0.003
42	Kaempferol rhamnoside hexoside	0.073 \pm 0.007	-	-
43	Isoquercitrin	-	-0	
41	Plantagoganidinic acid ^a	2.885 \pm 0.238	1.513 \pm 0.075	2.079 \pm 0.386
54	Unknown	0.054 \pm 0.009	0.022 \pm 0.005	0.016 \pm 0.001
57	Unknown	0.03		

3

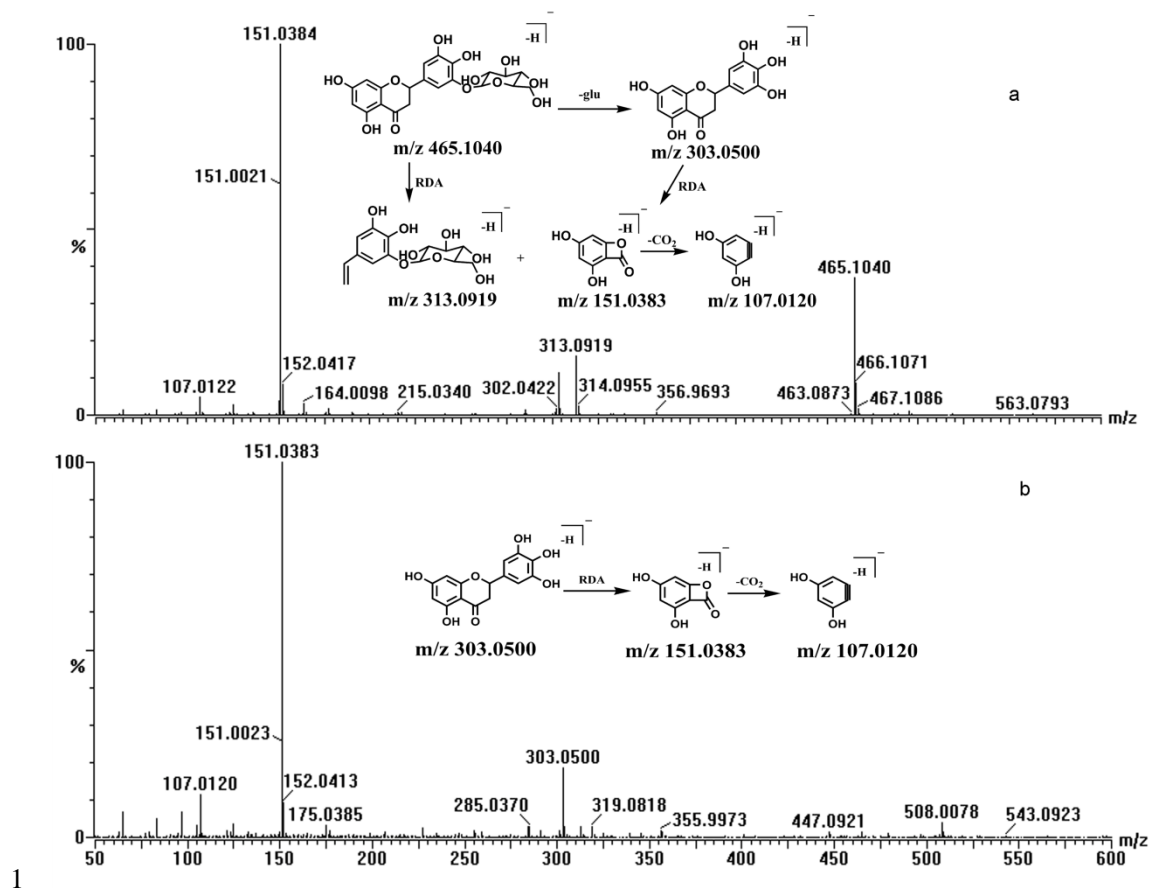
4



1
 2 **Figure 1** Base peak chromatograms of Plantaginis Semen obtained by UPLC/ESI-QTOF-MS. (a)
 3 *Plantago asiatica* L. in the negative ion mode. (b) *Plantago depressa* Willd. in the negative ion
 4 mode. (c) *Plantago major* L. in the negative ion mode.



2 **Figure 2** The proposed fragmentation pathway of plantamajoside.



1

2 **Figure 3** Fragmentation reactions of flavonoids. (a) plantagoside and (b) pentahydroxyflavanone.

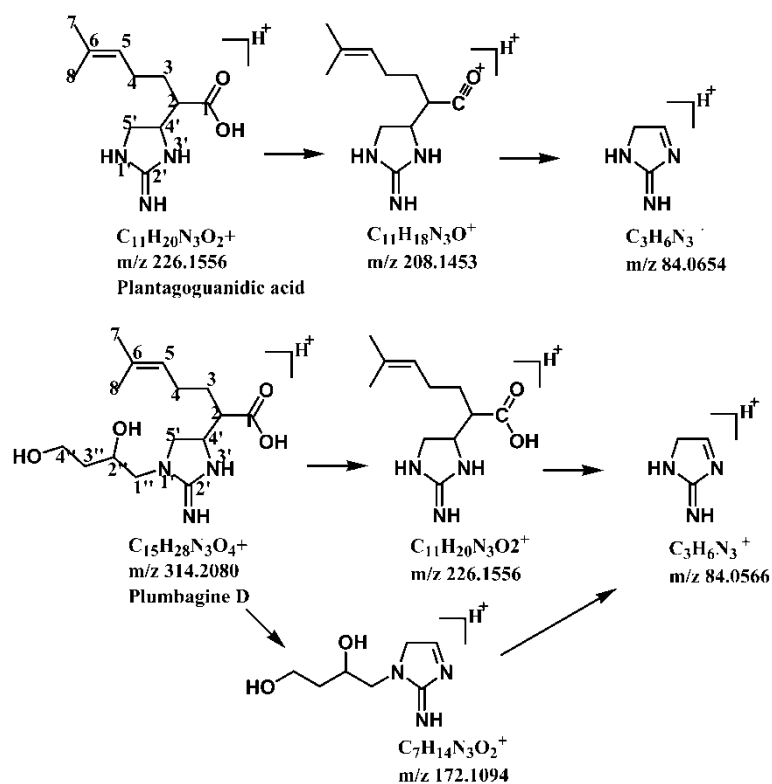


Figure 4 Fragmentation patterns of plantagoganidinic acid and plumbagine D.